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(57) Abstract

The present invention relates to a method of treatment of cancer with gene therapy to express a cytokine in tumor cells in combination therapy with a platin compound, in particular for the treatment of ovarian carcinomas. In a preferred aspect, the invention relates to a method for treating ovarian carcinomas, which includes a first treatment step utilizing cisplatin followed by a second treatment step of transfection with a cytokine cancer treatment gene/liposome complex, e.g. Interferonγ (IFNγ).

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CYTOKINE AND CISPLATIN-BASED GENE THERAPY OF CARCINOMAS

The present invention relates to a method of treatment of cancer with gene therapy to express a cytokine in tumor cells in combination therapy with a platin compound, in particular for the treatment of ovarian carcinomas.

A platin, cisplatin (cis-diaminodichloro platinum (II)), is a DNA cross-linking agent which is one of the few drugs routinely used in treating ovarian carcinoma. Cisplatin also activates macrophages in the presence of immuno-stimulant(s) to kill tumor cells directly or indirectly by producing nitric oxide (NO) and other tumoricidal factors. NO is an important cytostatic/cytotoxic mediator for tumor cell killing *in vitro* and *in vivo* by inhibiting TCA cycle, mitochondrial respiration, DNA synthesis, and DNA repair enzymes and by inducing apoptosis. Unfortunately, the clinical effectiveness of platins, such as cisplatin, is limited by the emergence of drug resistance in tumor cell populations.

Interferon γ (IFNγ) is an example of a gene which also activates macrophages to induce both NO-producing inducible nitric oxide synthase (iNOS) and tumor necrosis factor α (TNFα). High expression of the IFNγ gene locally in tumors can activate tumor-infiltrated macrophages and kill tumor cells or inhibit tumor growth effectively. Thus, transfection of tumor cells with a gene such as the IFNγ

gene can be a means for local production of high levels of proteins such as the IFNγ protein as a means for killing tumor cells and inhibiting their growth. As further examples of such proteins, expression of a major histocompatibility complex protein (class 1) alloantigen in the tumor cells has resulted in the induction of a tumor-specific cytotoxic T lymphocyte response in a host, leading to the inhibition of tumor growth and prolongation of the host survival. Direct injection of a DNA-liposome complex into the cutaneous melanoma lesions has also resulted in the alloantigen expression in the tumor cells and partial regression of the tumor in the treated individuals.

Cationic liposome-mediated transfection (lipofection) has been used for direct gene transfer *in vitro* and *in vivo*. Direct injection of a plasmid DNA-liposome complex into the tumor (*in situ* lipofection) has been reported in animal models and in clinical trials. For example, human ovarian carcinoma cells (*e.g.*, the ovarian 2008 carcinoma cell line) grown as a subcutaneous solid tumor in a severely combined immunodeficient (SCID) mouse can be transfected by *in situ* lipofection. Such lipofection results in slower tumor growth and carcinoma cell death. Thus, efficient transgene expression in the tumor by direct gene transfer can bring a highly desired therapeutic benefit to the host.

Lipofection may become an efficient method for such direct gene transfer *in vitro* and *in vivo* and result in an efficient means to provide transgene expression in cancer cells. Lipofection is emerging as an important, but still unperfected means to implement gene therapy in the treatment of cancer. Direct injection of a plasmid DNA/liposome complex into the tumor (*in situ* lipofection) has been utilized. Nevertheless, a number of tumors of animals are still not efficiently transfectable.

Accordingly, there is a need for a better method for treating cancers utilizing gene therapy, especially methods which result in higher levels of local expression of cytokine proteins such as the IFNγ protein. Therefore,

more effective methods for efficient transfection with such genes as part of an unexpectedly superior combination treatment protocol would be a significant advance towards such a treatment goal.

Summary of the Invention

According to the present invention, an enhanced combination treatment method for treating carcinomas is provided which utilizes gene therapy to express a cytokine in tumor cells after such tumor cells have been treated with a platin compound. In a preferred aspect, the present invention involves the treatment of carcinoma cells (such as ovarian tumor cells) with a composition such as a platin-based composition followed by incorporation of a cytokine cancer treatment gene into the carcinoma cells, preferably via lipofection to express such cytokine in the carcinoma cells. In a further preferred aspect, the invention relates to a method for treating ovarian carcinomas, which includes a step utilizing cisplatin treatment followed by a transfection step to provide a polynucleotide which encodes and expresses a cytokine , e.g. Interferonγ (IFNγ). In a preferred aspect said cytokine is encoded by a cytokine gene selected from the group consisting of an IFNγ gene, a TNFα gene, an iNOS gene or an interleukin gene. It has been found that unexpectedly superior carcinoma treatment will result from such a combination treatment protocol that is superior to the sum of the results of the individual carcinoma treatment methods (*i.e.*, combination treatment results are greater than (platin treatment results) + (cytokine gene expression treatment results)).

According to the invention tumor cells are first treated with an anti-cancer drug such as a platin (or cells are obtained which are observed to be sensitized or resistant to such a platin or similar compound) and then a cytokine gene is inserted into the tumor cells, preferably by *in vivo* lipofection. It has been observed that tumor treated with a platin, which may be a single treatment or

multiple treatments that may even render the cells sensitive or resistant to platin, when further treated to express a cytokine provides for increased expression over an increased period of time and a provides a more effective treatment of the cancer. For example, tumor cells treated with a platin such as *cis*-diaminedichloroplatinum(II) (cisplatin), are more susceptible to treatment with a cationic liposome-DNA complex than the non-treated parent cells. Thus, the tendency of ovarian cancer cells to readily develop low-level cisplatin resistance upon cisplatin injection into animals bearing tumors of such cancer cells can be exploited for the purposes of the present invention and such tumors utilized for transfection of the desired cytokine cancer treatment gene(s) into the cancer cells for the instant combination treatment method.

Accordingly, the present invention includes in a preferred embodiment method steps for enhancing tumor cell transfection and for providing a prolonged and high level of expression of an inserted cytokine cancer treatment gene. In particular, injection of cisplatin into the host can enhance the sensitivity of the tumor to the subsequent injection of a DNA-liposome complex (lipofection), wherein the DNA codes for the IFNγ gene or the like, or for a tumor suppressor gene. This is demonstrated, according to the invention, with human ovarian carcinoma cells grown in the severe combined immunodeficient (SCID) mouse.

According to a preferred embodiment, the present invention, the IFNγ gene is utilized, in combination with cisplatin, for its tumor killing potential based on its production of NO and TNFα. This preferred embodiment of the invention provides direct evidence that an aggressive syngeneic murine ovarian carcinoma which is poorly responsive to treatment with either the IFNγ gene alone or cisplatin alone, none-the-less when treated with cisplatin and subsequent lipofection with an IFNγ gene the responsive treatment is observed. This data shows that a single cisplatin injection can maintain not only the elevated

level of IFNγ for at least 3 weeks but also provide a long lasting expression of IFNγ without further cisplatin treatment. Indeed, such a surprisingly successful cisplatin-based gene therapy protocol can be very effective in treating the advanced ovarian cancer patients regardless of their previous exposure to cisplatin.

In another aspect the invention provides a method for treatment of a carcinoma comprising (a) a first treatment step of inserting a cytokine cancer treatment gene into carcinoma cells whereupon said gene is expressed therein; and (b) a second treatment step of treating carcinoma cells with a platin anti-cancer compound. In a preferred embodiment said cytokine is encoded by a cytokine gene selected from the group consisting of an IFNγ gene, a TNFα gene, an iNOS gene or an interleukin gene. Particularly preferred is the cytokine encoded by an IFNγ gene. In another preferred aspect of the method according to the invention, the carcinoma cells are resistant to treatment with a platin prior to insertion the cytokine gene.

In another embodiment of the invention, the invention provides a method for removing the resistance of carcinoma cells towards treatment with an anti-cancer compound comprising inserting into said cancer cells at least one cytokine gene and the expressing said gene in said cancer cells. Such a method renders carcinoma cells again susceptible to treatment with an anti-cancer compound such as a platin (particularly cisplatin). Moreover, the removal of the resistance to the anticancer compound is long-term and appears to be a permanent loss of resistance. Particularly preferred is a method wherein the cytokine is encoded by a cytokine gene selected from the group consisting of an IFNγ gene, a TNFα gene, an iNOS gene or an interleukin gene. Further preferred is a method wherein the cytokine is encoded by an IFNγ gene. In still another preferred aspect of the method according to the invention, the carcinoma cells are resistant to treatment with a platin prior to insertion the cytokine gene.

The effectiveness of the combination of cisplatin treatment and cytokine gene therapy results in enhanced expression of the cytokine gene, which in turn, increases the activation of macrophages to enhance the production of NO and TNFα for tumor cell killing, and can result in synergistic cooperation between the actions of NO and TNFα in killing tumor cells. NO can regulate the activation of NFkB and the suppression of NFkB activation increases susceptibility of tumor cells to TNFα-induced apoptotic cell death. Apoptotic activity was observed in NO-dependent inhibition of NFkB activation and TNFα-mediated activation of caspase-family activity in the ascites of animal treated with both cisplatin and IFNγ gene.

Therefore, cisplatin-based gene therapy may be a used for many different types of cancer gene therapy, preferably with other cytokines (*e.g*, TNFα and interleukins). Moreover, other anticancer drugs that sensitize tumor cells in a manner similar to cisplatin can be combined with lipofection cytokine gene therapy to provide the combination treatment protocol of the present invention.

The present method of combination platin treatment and gene therapy surprisingly demonstrates complete killing of ovarian tumor cells with about 92% survival for at least 80 days by prior cisplatin treatment-based IFNγ gene therapy through enhanced long-lasting production of NO with TNFα in only tumor localized sites.

Brief Description of Figures

Figs. 1A-1D show the expression of transgenes in MOT ascites, thioglycollate-induced macrophages, liver and lung. As shown in Fig. 1A, cisplatin-treated ascites only expressed 45 to 50% of CAT transgene when a complex of pUCCMVCAT plasmid DNA/liposomes was i.p. injected. The ascites of animals treated with either cisplatin or CAT DNA/liposome complex alone were not transfectable. Since macrophages are major cells residing in the peritoneum, it is possible that the ascites affected by cisplatin were not the tumor cells but macrophage cells. Thus, macrophages

were induced with thioglycollate in the peritoneum of mice and then exposed to cisplatin followed by transfection with a CAT DNA/liposome complex following a cisplatin injection. As shown in Fig. 1A, CAT gene did not express in the peritoneal macrophages, liver, or lung of cisplatin injected animals. This result indicates that the pre-treatment effects of cisplatin are specific to the tumor cells.

Fig 1B shows similar cisplatin pretreatment effects on tumor cells subjected to a therapeutic gene pBCMGNeo-IFNγ plasmid DNA. Surprisingly, the ascites of cisplatin-treated animals produced a continued high level of IFNγ (13,000 to 15,000 units/ml of ascitic fluid) after lipofection with IFNγ plasmid DNA, being compared to the endogenous level (approximately 1000 units).

Fig. 1C shows the IFNγ expression level in the tumor, and illustrates a series of *in situ* lipofections that were performed with respect to time after injection of cisplatin or the IFNγ DNA/liposome complex. The lipofection efficiency significantly enhanced at day 3.5 and reached a peak at day 11 and then declined thereafter after cisplatin administration.

Fig. 1D shows that IFNγ expression is prolonged over at least 1 week after a single lipofection following a cisplatin injection. Although animals were pre-treated with cisplatin, injection of plasmid DNA without liposomes did not express either CAT or IFNγ gene in ascitic tumors.

Figs. 2A and 2B show nitrate levels and TNFα levels, respectively, to illustrate the effect of enhanced expression of IFNγ gene on the production of NO and TNFα which have a synergistic cooperation for tumor cell killing, and to show results of the measured amounts of NO and TNFα in the tumor. Only mice receiving the combined treatment of cisplatin and IFNγ produced significant amounts of NO, measured as nitrite plus nitrate level (Fig. 2A), and TNFα (Fig. 2B). EPR signal of iron-nitrosyl complexes confirmed that NO was significantly produced in

the ascitic tumor cells of animals treated with both cisplatin and IFNγ DNA/liposome complex.

Figs. 3A and 3B illustrate survival of mice in five different treatment groups and show the results of tests to determine whether the cisplatin-based IFNγ gene therapy protocol is effective in treating a metastatic MOT ascitic tumor. Mice receiving IFNγ DNA/liposome complex once every 5 days for 4 weeks following a single injection of cisplatin survived for at least 80 days with a free of tumors, although mice were maintained without further treatment for 2 months (Fig. 3A). The tumor growth in peritoneum, lung, liver and spleen was examined and there was no detectable tumors observed (data not shown). All mice receiving the empty expression vector, cisplatin or IFNγ DNA/liposome complex alone had a pronounced abdominal mass at day 15 (animal 1 in Fig. 3B) and died in 15 to 26 days after MOT transplantation (Fig. 3A). Mice treated with cisplatin and IFNγ DNA without liposomes died in 19 to 25 days, indicating that liposomes were required for IFNγ production as shown in Fig. 1C and 1D.

Fig. 4 shows a comparison of resulting nitrite plus nitrate for several complexes *in vivo* in a mouse. All complexes contain liposomes. The complexes are: (1) CisPtPBS, (2) CisPt/Empty vector, (3) CisPt/IFNα-Expression Vector, and (3) CisPt with IFNα (bare DNA). Nitrite + Nitrate levels in μM are plotted against time post injection in Figure 4. Only the mice who were injected with the CisPt/IFNα-Expression vector liposome complex.

Detailed Description of the Invention

Tumors of cisplatin injected animals were significantly transfectable with a cytokine cancer treatment gene/liposome complex. The IFNγ expression was significantly enhanced at day 4 and reached a peak at day 11 and then declined thereafter, after cisplatin injection. The expression level prolonged over 1 week after a single lipofection following a cisplatin treatment. Both TNFα expression and nitric oxide production were also significantly elevated. Such tumor cell death by the

combined treatment of cisplatin and IFNγ may be to some extent related to the general mechanism of apoptosis. Data was obtained to determine whether complete inhibition of tumor growth or tumor cell killing by the combination therapy of Figs. 3A-3B is, in fact, due to a cytotoxic NO molecule and/or TNFα. Tumor bearing mice were treated with an IFNγ DNA/liposome complex and NOS inhibitor aminoguanidine simultaneously following a cisplatin injection. Mice, treated with 100 mg/kg of aminoguanidine which is a non-toxic dose to normal mice, died in 22 to 51 days, which indicates that the cytotoxic activity was inhibited by iNOS inhibitor. Thus, the antitumor effect of the combined cisplatin and IFNγ gene therapy can be attributed in a large part to enhanced levels of NO, a major effector for macrophage-mediating tumor cell killing.

Not only is the activity of CPP32 (Caspase-3 protein, an apoptosis (programmed cell death)-inducing protein) greatly increased but cleavage of 116 kD poly-ADP-ribosyl polymerase (PARP), a biosubstrate of CPP32-like protease, to 85 kD fragment occurs only in the tumors treated with both cisplatin and IFNγ gene. This observation is confirmed by DNA fragmentation, a characteristic of apoptosis, of tumor cells. The activation of apoptotic machinery by cisplatin-based IFNγ gene treatment is completely attenuated by the administration of nitric synthase inhibitor, NMA, and TNFα-neutralizing antibody. Further, the combination therapy of chemotherapeutic agents (cisplatin in particular) and a series of apoptosis-inducing genes also enhance therapeutic efficacy by increasing the expression of these genes in tumor localized sites. Finally, mice treated with both cisplatin and IFNγ gene survived for at least 80 days and were free of tumors, while mice receiving only one of either the IFNγ gene or cisplatin died in 15 to 26 days after tumor transplantation. Thus, anti-carcinoma cytokine gene therapy, such as IFNγ gene therapy, following cisplatin treatment is, in fact, an effective treatment method for the treatment of cancers particularly of ovarian cancers.

Moreover, treatment with a cytokine gene prior to treatment with anti-cancer compounds such as platins (in particular cisplatin) enhances the anti-cancer effects of such compounds. Additionally, treatment of platin resistant tumor cells (cells upon which the platin no longer has as effective an anti-cancer action) with a cytokine gene then renders the cells susceptible to the anti-cancer action of the platins (particularly cisplatin). Such an effective reversal of the platin-resistance is an important and unexpected discovery of the present invention.

Treatment agents other than cisplatin may be utilized with the invention, for example other platins, methotrexate, etoposide, cytosine arabinonucleoside, doxorubicin and vincristine. However, the dosage amounts and the periods for treatment of tumor cells with agents other than cisplatin will vary and may be optimized.

In addition to preferred 3ß[N-(N',N'-dimethylamino-ethane)carbamoyl]cholesterol/dioleoylphosphatidylethanol-amine (DC-chol)/(DOPE) cationic liposomes for lipofection according to the invention, other liposomes such as commercially available Lipofectamine and Lipofectin can be utilized according to the present invention. The DNA/liposome ratio in complexes for treatment of solid carcinoma tumors varies from 1:2, 1:1 or 2:1, but the preferred ratio is 1:1. With such solid tumors the injection volume to a small size tumor is limited. However, the peritoneum cavity of tumor-bearing mice is large enough to inject the injection volume of about 1 ml. Therefore, the DNA dose can be increased up to 1 mg or more with a ratio from 1:10 to 1:0.25. Thus, even though the *in vitro* lipofection preferred range is 1:10 to 1:20, the *in vivo* lipofection preferred ratio for solid tumors is 1:1. Further, the preferred ascites lipofection ratio is from 1:10 to 1:0.25.

Tumors which may be treated in accordance with the present invention include malignant primary and metastatic tumors. Such tumors which may be treated include, but are

not limited to, cancers which may be found in the oral epithelium, including, but not limited to, squamous cell carcinomas of the mouth, oral cavity, and upper aerodigestive tract, including the floor of the mouth, tongue, cheek, gums, or palate, adenocarcinoma of the oral cavity, lip cancers, Kaposi's sarcoma, and laryngeal papillomas and nasopharyngeal cancers which may have spread to the oral epithelium; tumors occurring in the adrenal glands; bladder, bone; breast; cervix; endocrine glands (including thyroid glands, the pituitary gland, and the pancreas); stomach; small intestine; peritoneal cavity; colon; rectum; heart; hematopoietic tissue; kidney; liver; lung; muscle; nervous system; brain; eye; oral cavity; pharynx; larynx and other head and neck cancers; ovaries; penis; prostate; skin (including melanoma, basal cell carcinoma, and squamous cell carcinoma); testicles; thymus; and uterus. The present invention is applicable particularly to the treatment of ovarian carcinomas.

In the non-limiting examples below, a preferred embodiment of the present invention utilizes as the treatment agent cis-diaminedichloro platinum (II) and involves *in vivo* lipofection of tumor cells with the cytokine cancer treatment gene, interferon γ (IFNγ). A murine metastatic ovarian carcinoma model with human carcinoma cells was utilized to illustrate this embodiment of the invention. The expression of IFNγ gene in ascitic tumors reached a peak at day 11 (regarded in the Figures as the 100% point) after cisplatin treatment and lasted over 1 week. A local increase in IFNγ gene expression increased a local expression of inducible nitric oxide synthase and tumor necrosis factor α gene. This treatment suppressed tumor growth (or killed tumor cells) and increased a long-term survival of animals with a free of tumors, which was prevented by simultaneous treatment of iNOS inhibitor aminoguinidine. The results indicate that the cisplatin-based IFNγ gene therapy is an effective treatment method and nitric oxide may now be utilized to play a major part

in tumor cell killing or growth in cancer treatment protocols.

In the examples below, cisplatin may be purchased from Sigma. Thioglycollate may be purchased from Difco, Inc. (Detroit, MI). Aminoguanidine hydrochloride was from Aldrich (Milwaukee, WI). TNFα antibody was from R&D Systems (Minneapolis, MN). [^{14}C] chloramphenicol was obtained from ICN (Irvine, CA). MOT can be obtained from Dr. F.M. Sirotnak, Memorial Sloan Kettering, NY, NY. A murine germ-cell tumor MOT arose spontaneously in and syngeneic for a C3HeB/FeJ mouse with rapid growth as malignant ascites. Five-to-six-week-old female C3H mice (C3HeB/FeJ, Jax Lab., Bar Harbor, MA) weighing 23-25 g were used in all experiments. Animal care was in accordance with the institutional guidelines.

Example 1

Preparation of Plasmid DNA and Plasmid/Liposome Complexes

The bacterial chloramphenicol acetyltransferase (CAT) as a reporter gene was used to measure expression levels in ascitic tumors, the peritoneal macrophages, liver and lung. Murine IFNγ was utilized as a therapeutic gene to measure expression levels in ascitic tumors and its therapeutic effect. A pUC18-based plasmid, pUCCMVCAT (5.1 kb) was utilized which contains the full-length CAT cDNA downstream from the immediate early promoter of the human cytomegalovirus element (available from H. Farhood and N. Serbina, University of Pittsburgh, Pittsburgh, PA). A mIFNγ cDNA expression vector (pBCMGNeo-mIFNγ) (15.4 kb) was constructed by inserting cDNA-encoding murine IFNγ into the bovine papilloma virus-based plasmid BCMGNeo (available from Dr. D. Pardoll, Johns Hopkins University, Baltimore, MD). The plasmids were purified according to the Sambrook *et al.* textbook.

Cationic liposomes were composed of 3β(N-(N',N'-dimethylaminoethane)carbamoyl)cholesterol(DC-chol) and dioleoyl phosphatidylethanolamine, 3-2 (mol/mol).

pUCCMVCAT (30 μg in 30 μl of 1x TE buffer (10 mM Tris-HCl, pH 8.0/1 mM EDTA) was diluted with 100 μl of 5% (wt/vol) dextrose and then mixed with 30 nmol of DC-chol-dioleoyl phosphatidylethanolamine liposomes in 15 μl of 20 mM Hepes (pH 7.8).

Example 2

In situ lipofection

Solid tumors were established from the 2008 human ovarian carcinoma cell lines (available, for example, from Paul A. Andrews, Georgetown University, Washington, D.C.). The 2008 cells were seeded in 150-mm tissue culture plates and grown to confluency in RPMI 1640 medium (GIBCO/BRL) supplemented with 10% (vol/vol) heat-inactivated fetal bovine
serum, penicillin (100 unit/ml), and streptomycin (100 μg/ml). Cells at 30-40% confluency were harvested by treatment with trypsin/EDTA (GIBCO/BRL), washed with complete medium, and resuspended in sterile PBS at 2 x 10^6 cells per ml. From this suspension, 0.1 ml was injected s.c. to the flanks and back of SCID mice by using a 21-gauge needle.

MOT ascites (2 x 10^6 cells/0.2 ml) were injected into the peritoneum of C3H mice to induce the formation of ascitic tumor using a 21-gauge needle. Seven days after tumor transplantation, when the tumors were about 8 mm in diameter, a single dose of cisplatin (8 mg/kg) dissolved in PBS was injected i.p. into mice. One week later, mice were injected with DNA-liposome complexes. Such complexes are formed as described above. About 10 minutes after its formation, this complex was injected directly into the tumor in three sites. Cationic DC-chol liposomes were composed of DC-chol, 3β[N'N'-dimethyl aminoethane) carbamoyl] cholesterol (DC-chol) and dioleoyl phosphatidylethanolamine, 3:2 (mol/mol). 200 μg of plasmid DNA in 1x TE buffer (1 μg/μl) was mixed with 200 nmole of DC-chol liposome in water (2 nmol/μl). Then this complex was injected directly into the peritoneum of MOT-bearing

mice using a 27-gauge needle. The animals were sacrificed 3 days later to examine the expression of genes.

Example 3

Assays

Radiometric assay of CAT activity.

The ascitic tumors taken from animals were homogenized, in 40 mM Tris-HCl, pH 7.5/10 mM EDTA/150 mM NaCl. After homogenization, cells were lysed by three freeze-thaw cycles, and the lysate was heated at 65°C for 10 minutes and centrifuged at 16,000 x g for 10 minutes. The protein concentration of the extracts was measured with a Coomassic blue G250-based assay (Bio-Rad). The protein extract of each tumor (100 μg) was then assayed for the CAT activity using [^{14}C]chloramphenicol as a substrate. The acetylated and nonacetylated forms of chloramphenicol were quantified by Phosphoimager (Molecular Dynamics). CAT activity was expressed as % conversion, acetylated forms divided by acetylated plus nonacetylated forms.

IFNγ assay.

This was performed as described previously. RAW 264.7 (1x 10^5 cells/200 μl/well) were incubated for 4 to 6 h and then incubated with a serially diluted ascitic fluid in culture media and a standard mIFNγ (0-100 units/ml). After 24 h incubation at 37°C in CO_2 incubator, the media was collected for measurement of nitrite accumulation. Culture medium (100 μl) were mixed with an equal volume of Griess reagent (1 part 0.1% naphthylethylenediamine dihydrochloride in water plus 1 part 1% sulfanilamide in 5% H_3PO_4) in a 96 well-plate. Nitrite concentration was measured at 550 nm using a microplate reader. IFNγ concentration was calculated from nitrite concentration using the standard curve with mIFNγ.

Determination of NO synthesis *in vivo*.

The NO concentration in ascitic fluids was measured by HPLC as described previously. Protein was precipitated with double volume of 0.5 N NaOH and 10% $ZnSO_4$. Total NO_2^-

plus NO_3^- in the ascites was determined by converting the NO_3^- to NO_2^- using an automated HPLC.

TNFα assay.

The ascites taken from animals were centrifuged at 12,000 rpm for 20 min. at 4°C to obtain ascitic fluids. TNFα in ascitic fluids was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems) using TNFα antibody according to the manufacturer's protocol.

Example 4

Gene therapy in the MOT tumor model

MOT cells (2 x 10^6/0.2 ml ascites) were transplanted i.p. into each animal and CAT was utilized as a reporter gene. After 4 days, mice were i.p. treated with either phosphate buffered saline (PBS) or cisplatin (8 mg/kg). IFNγ treatment was initiated 5 days after cisplatin injection to allow for increase in NO synthesis. Either the empty vector pBCMGNeo (200 μg)/DC-chol liposome (200 nmol) or pBCMGNeo-IFNγ DNA (200 μg)/DC-chol liposome (200 nmole) complexes were injected i.p. once every 5 days for 4 weeks. After that, the mice were maintained without further treatment. Since tumor-bearing mice all die when their body weight reaches about 42 g, a mouse with 40g of body weight was considered as a dead point and thus sacrificed. Some animals were, every 2 days for 2 weeks and every 3 days for next 2 weeks, injected i.p. with 100 mg/kg of NOS inhibitor aminoguanidine 1 day after injection of a DNA/liposome complex.

To examine the expression level of CAT gene in ascites, mice bearing tumors were injected i.p. with either PBS or cisplatin (8 mg/kg). To examine the expression level of CAT gene in macrophages, liver and lung, mice were injected i.p. with 1 ml of 3% thioglycollate broth for induction of macrophages and, 3 days later, with cisplatin (8 mg/kg) for the pre-treatment agent. After 1 week, the mice were injected i.p. with either PBS or a complex of plasmid pUCCMVCAT (200 μg) and DC-chol liposome (200 nmole). Animals were sacrificed 3 days later and the protein extracts from tumors, macrophages, liver and lung

were then assayed for CAT activity. Results are reported in Fig. 1A.

IFNγ gene expression was determined by injecting pBCMGNeo-IFNγ (200 μg) with or without liposome (200 nmole) i.p. into tumor-bearing mice as described above. IFNγ activities in the ascites collected were measured and results are reported in Fig. 1B.

The time course of IFNγ expression after cisplatin injection was determined utilizing mice bearing MOT tumors which were injected i.p. with cisplatin (8 mg/kg). After various periods, the tumor was injected i.p. with 200 μg of pBCMGNeo-IFNγ plasmid DNA alone or a complex containing 200 μg of DNA and 200 nmol of DC-chol liposome. The ascites were taken three days later and processed for IFNγ assay. The results are reported in Fig. 1C.

The time course of IFNγ expression after lipofection was determined utilizing mice bearing MOT tumors which were injected i.p. with cisplatin (8 mg/kg). One week later, the tumor was injected i.p. with 200 μg of pBCMGNeo-IFNγ plasmid DNA alone or a complex containing 200 μg of DNA and 200 nmol of DC-chol liposome. At different time points, the ascites were taken and processed for IFNγ assay. The level of IFNγ expression at day 11 was considered as 100%. The results are reported in Fig. 1D. Each data point is the mean ± s.d. from 3 animals.

The enhanced production of NO and TNFα resulting from the enhanced expression of IFNγ gene was determined utilizing mice bearing MOT tumor were injected i.p. with PBS or cisplatin (8 mg/kg). One week later, the mice were injected i.p. with either the empty vector pBCMGNeo or pBCMGNeo-IFNγ (200 μg)/liposome (200 nmole) complex. Three days later, the ascites were collected for the measurement of NO reported in Fig. 2A and TNFα reported in Fig. 2B.

The enhanced antitumor activity of the combined treatment of MOT ascitic tumors with cisplatin and IFNγ DNA was determined utilizing mice bearing MOT. MOT cells ($2x10^6$/0.2 ml ascites) were transplanted into the peritoneum of C3H mice. After 4 days, mice were treated

i.p. with either PBS or cisplatin (8 mg/kg). Five days later, mice were i.p. injected with either the empty vector or IFNγ plasmid DNA (200 μg) with liposomes (200 nmole) once every 5 days for 4 weeks and then mice were maintained without further treatment. Animals were divided into 5 groups. Group 1 received PBS and the empty vector pBCMGNeo DNA/liposome complex, Group 2 received PBS and pBCMGNeo-IFNγ DNA/liposome complex, Group 3 received cisplatin and pBCMGNeo DNA/liposome complex, Group 4 received cisplatin and pBCMGNeo-IFNγ DNA/liposome complex, and Group 5 received cisplatin, pBCMGNeo-IFNγ DNA/liposome complex and aminoguanidine. 100 mg/kg of aminoguanidine was, every 2 days for the first 2 weeks and 3 days for the next 2 weeks, injected i.p. 1 day after injection of a DNA/liposome complex. Twelve mice per each group was used (n=12). The results are shown in Fig. 3A. Figure 3B is a photo of C3H mice bearing MOT tumors. Photo was taken at day 20 after transplantation of MOT cells. Animal 1 and 2 represent Group 3 and 4, respectively.

EXAMPLE 5

Lipofection *in vitro*

Solid tumors were established from the 2008 human ovarian carcinoma cell lines as described in Example 2, above. Cells at 30-40% confluency were harvested, washed with complete medium, and resuspended in sterile PBS at 2 x 10^6 cells per ml.

Plasmid pUCCMVCAT in 1x TE buffer (0-2.0 μg) was mixed gently with 10 nmol of DC-chol-dioleoyl phosphantidyl-ethanolamine liposomes at room temperature in 1 ml of serum-free RPMI 1640 medium, incubated for 10 minutes, and then added to the cell suspensions described above. The DNA-liposome complex was added to the cells and incubated at 37°C in 5% C)$_2$/95% air for 6 hours. Transfection medium in the growth medium containing 10% fetal bovine serum for 48 hours before the CAT assay as performed.

WHAT IS CLAIMED IS:

1. A method for treatment of a carcinoma comprising:

(a) a first treatment step of treating carcinoma cells with a platin anti-cancer compound, and

(b) as second treatment step of inserting a cytokine cancer treatment gene into the treated carcinoma cells, which is expressed therein.

2. A method according to claim 1, wherein said first step treatment agent is cisplatin.

3. A method according to claim 1, wherein said cytokine gene is selected from the group consisting of an IFNγ gene, a TNFα gene, an iNOS gene or an interleukin gene.

4. A method according to claim 3, wherein said cytokine gene is an IFNγ gene.

5. A method according to claim 1, wherein said gene is inserted into tumor cells via lipofection.

6. A method according to claim 5, wherein said gene is inserted via lipofection with a cationic liposome which is selected from the group consisting of Lipofectin (a), Lipofectin (b) and DC-Chol/DOPE.

7. A method according to claim 6, wherein said cationic liposome is DC-Chol/DOPE.

8. A method according to claim 5, wherein said lipofection gene insertion is conducted with a lipofection complex wherein the DNA of the cytokine cancer treatment gene and the liposome are present in a 1:2 to 2:1 ratio.

9. A method according to claim 8, wherein said ratio is a 1:1 ratio.

10. A method according to claim 9, wherein said gene insertion is an *in vivo* gene insertion.

11. A method for treatment of a carcinoma comprising:

(a) a first treatment step of inserting a cytokine cancer treatment gene into carcinoma cells whereupon said gene is expressed therein; and

(b) a second treatment step of treating carcinoma cells with a platin anti-cancer compound.

12. A method according to claim 11, wherein said carcinoma cells are resistant to treatment with a platin prior to inserting the cytokine gene.

13. A method for removing the resistance of carcinoma cells towards treatment with an anti-cancer compound comprising inserting into said cancer cells at least one cytokine gene and expressing said gene in said cancer cells.

14. A method according to claim 13 wherein the resistance of said carcinoma cells which is removed is a resistance to a platin compound.

15. A method according to claim 11, wherein said second treatment step treatment agent is cisplatin.

16. A method according to claim 11, wherein said cytokine gene is selected from the group consisting of an IFNγ gene, a TNFα gene, an iNOS gene or an interleukin gene.

17. A method according to claim 16, wherein said cytokine gene is an IFNγ gene.

18. A method according to claim 13 wherein said cytokine gene is selected from the group consisting of an

IFNγ gene, a TNFα gene, an iNOS gene or an interleukin gene.

19. A method according to claim 18, wherein said gene is an IFNγ gene.

20. A method according to claim 14, wherein said carcinoma cells are ovarian carcinoma cells.

FIG. 1A

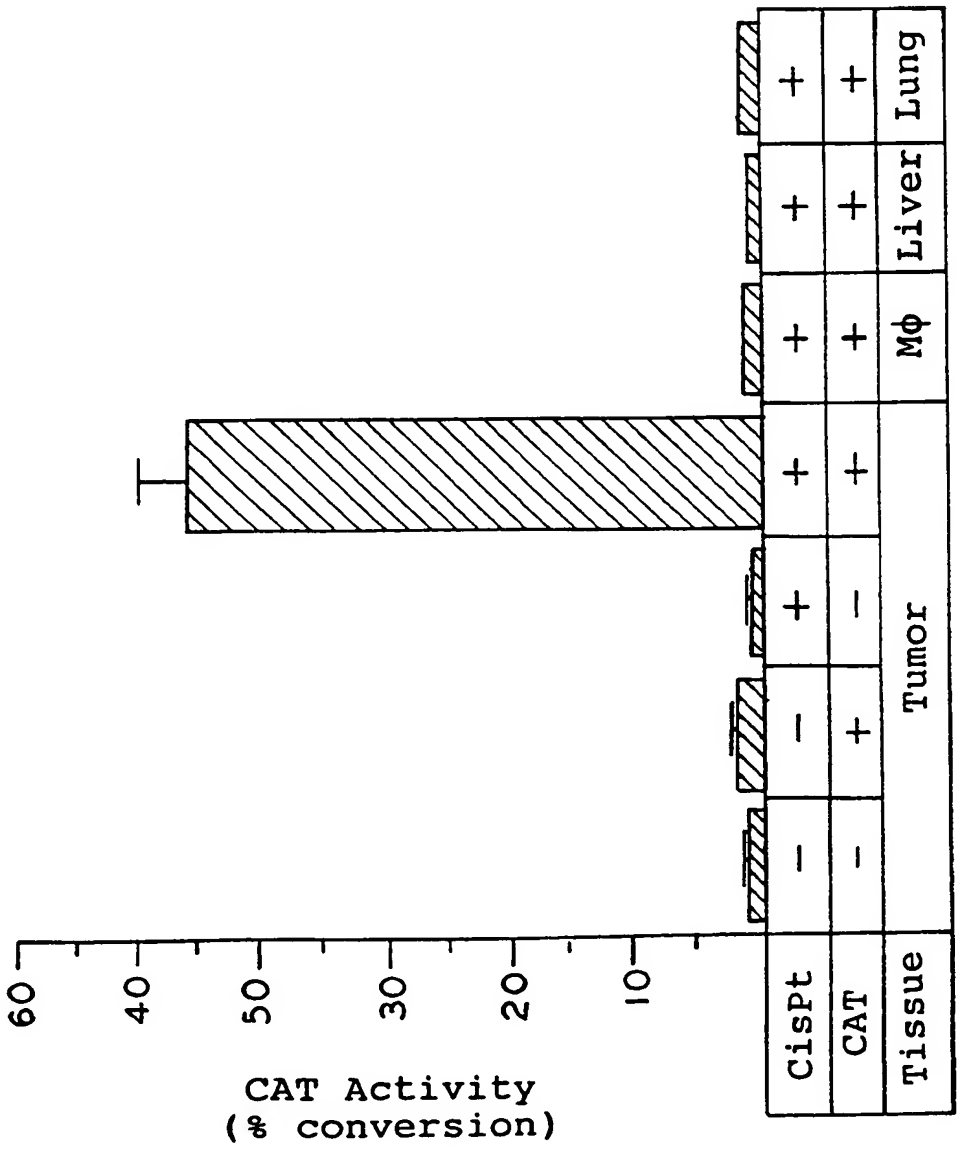

60
40
50
30
20
10
CAT Activity
(% conversion)
Tissue
CAT
CisPt
Tumor
Mϕ
Liver
Lung
-
+
-
+
-
+
-
+
-
+
-
+
-
+
-
+

FIG. 1B

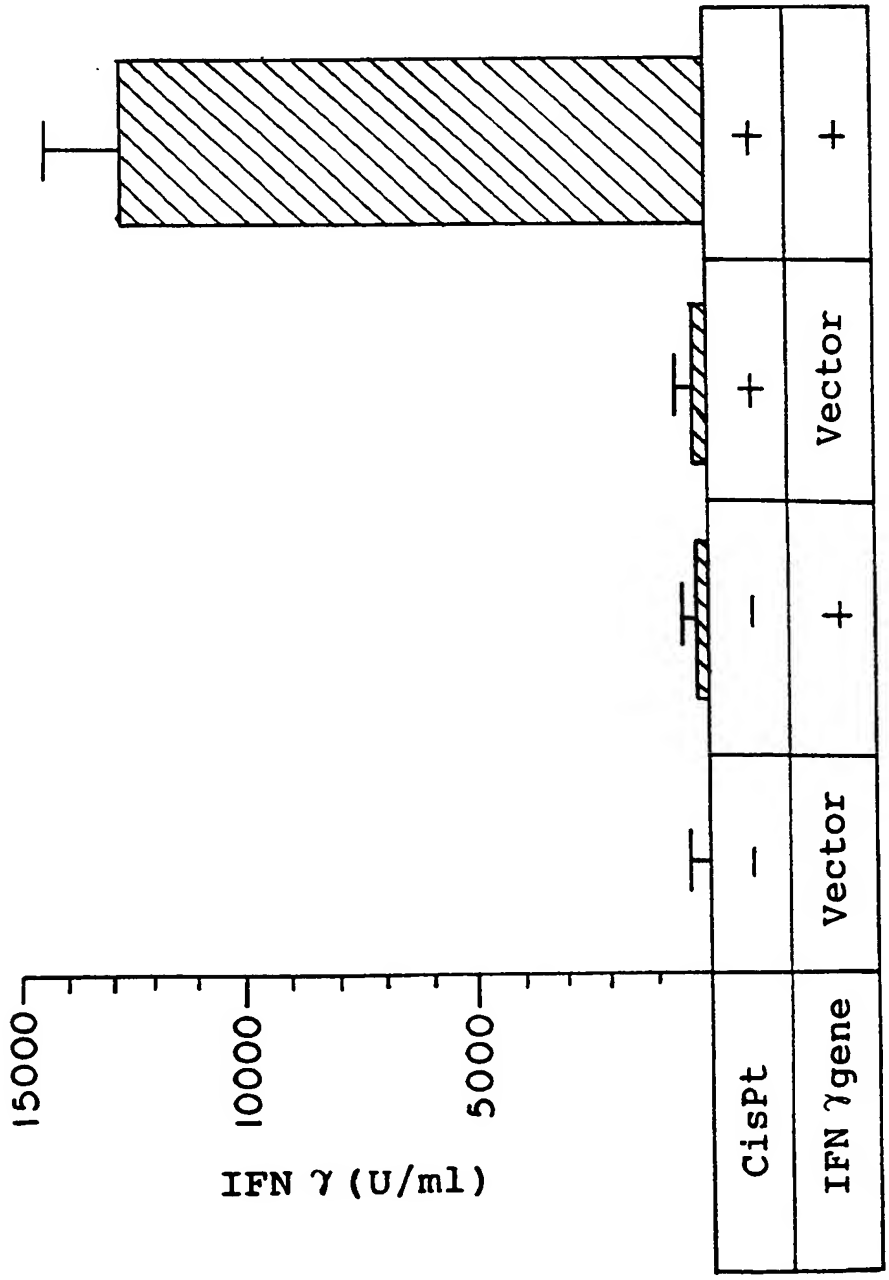


FIG. 1C

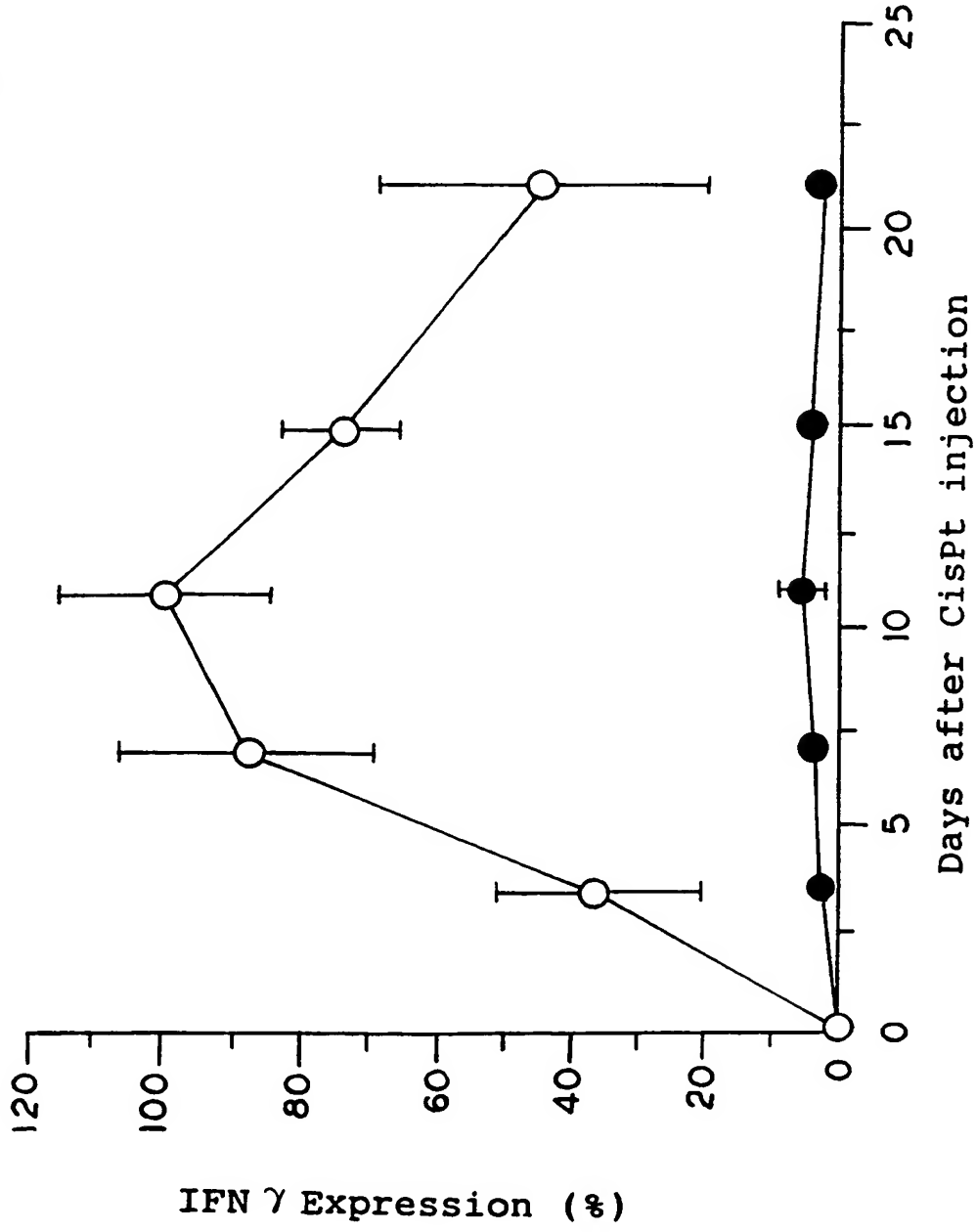

120
100
80
60
40
20
0
0
5
10
15
20
25
Days after CisPt injection
IFN γ Expression (%)

FIG. 1D

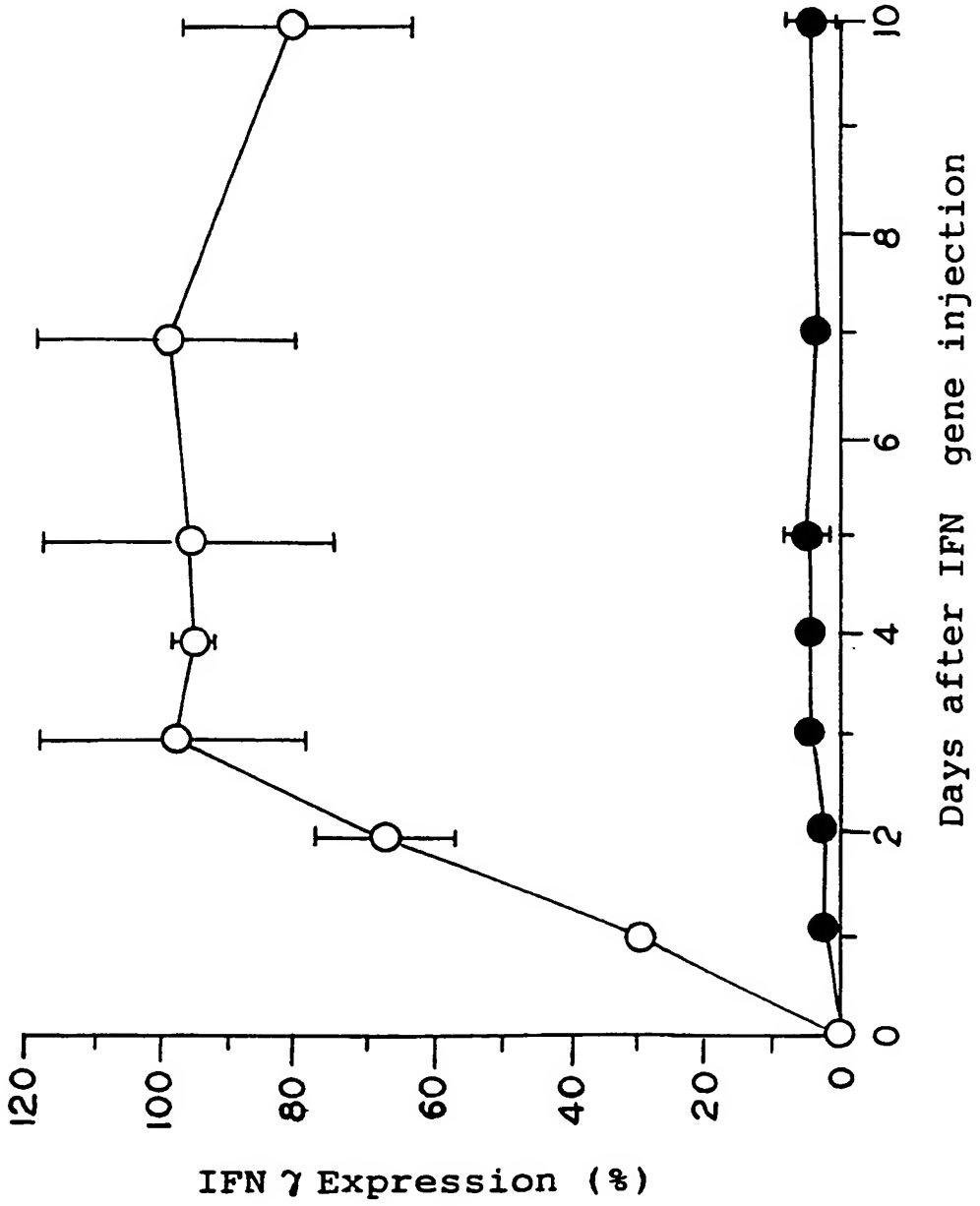

120
100
80
60
40
20
0
IFN γ Expression (%)
0
2
4
6
8
10
Days after IFN gene injection

FIG. 2A

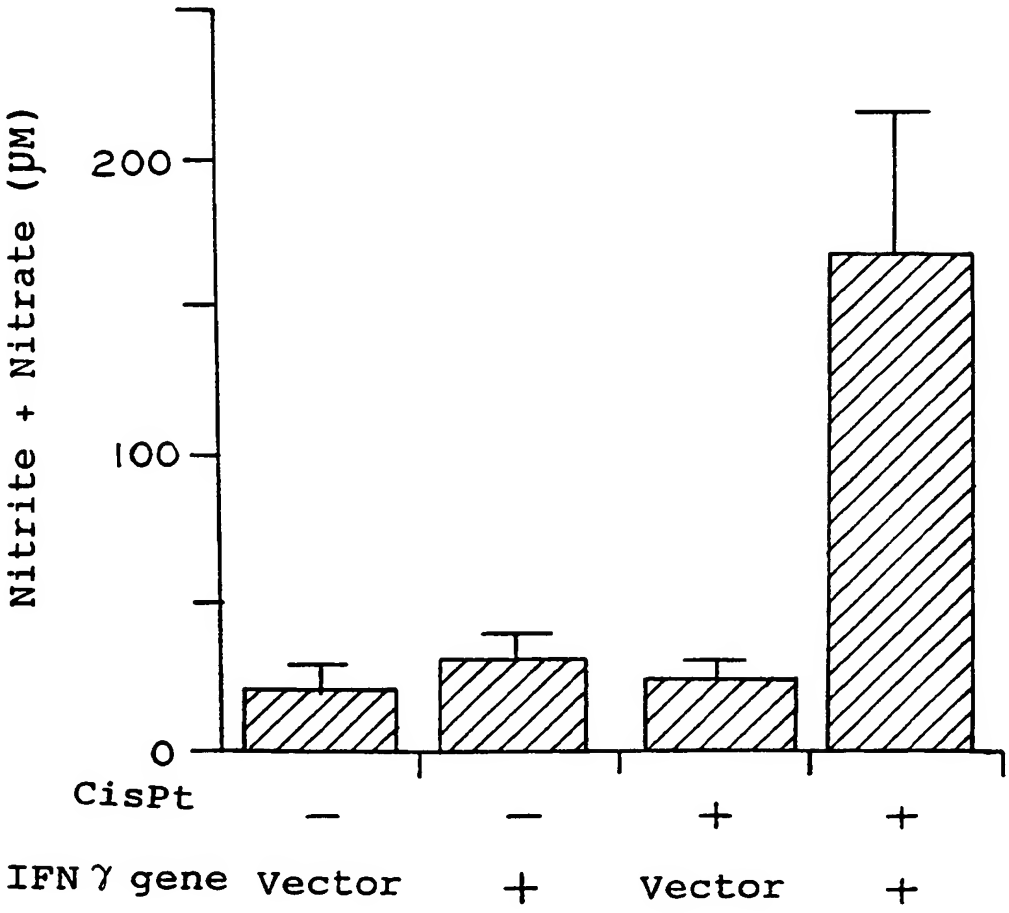


FIG. 2B

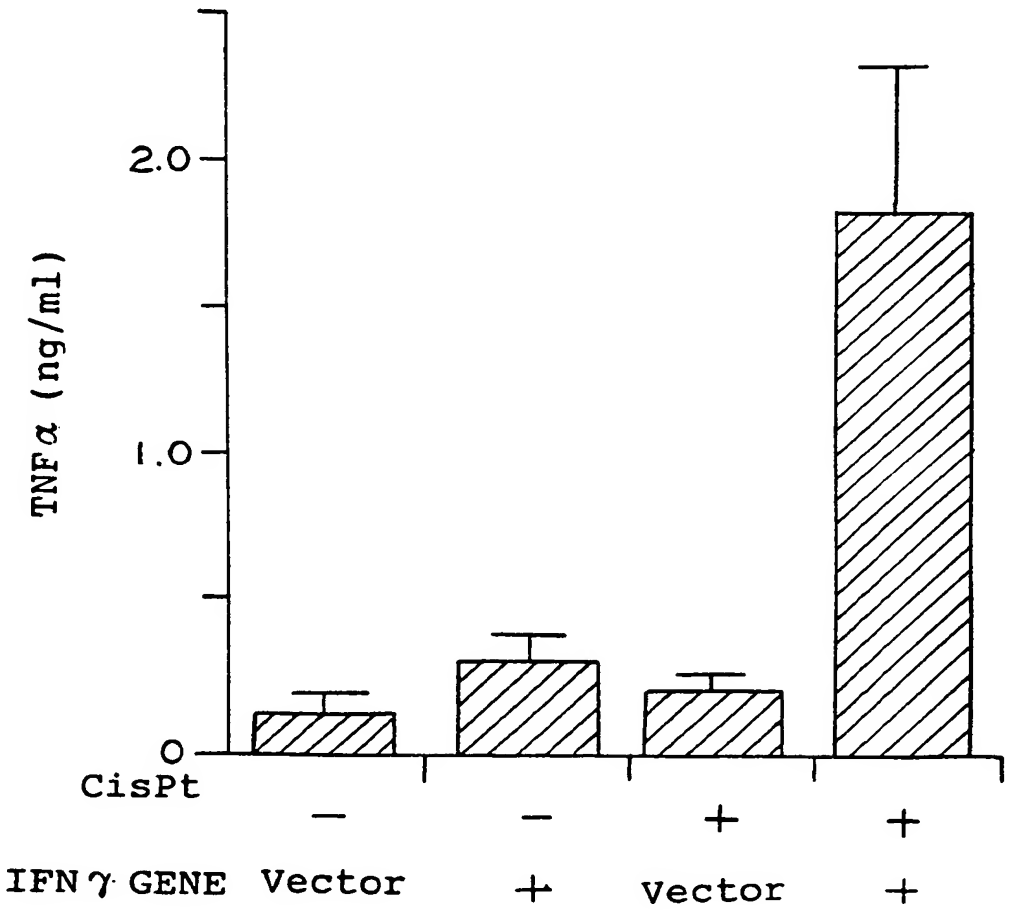


FIG. 3A

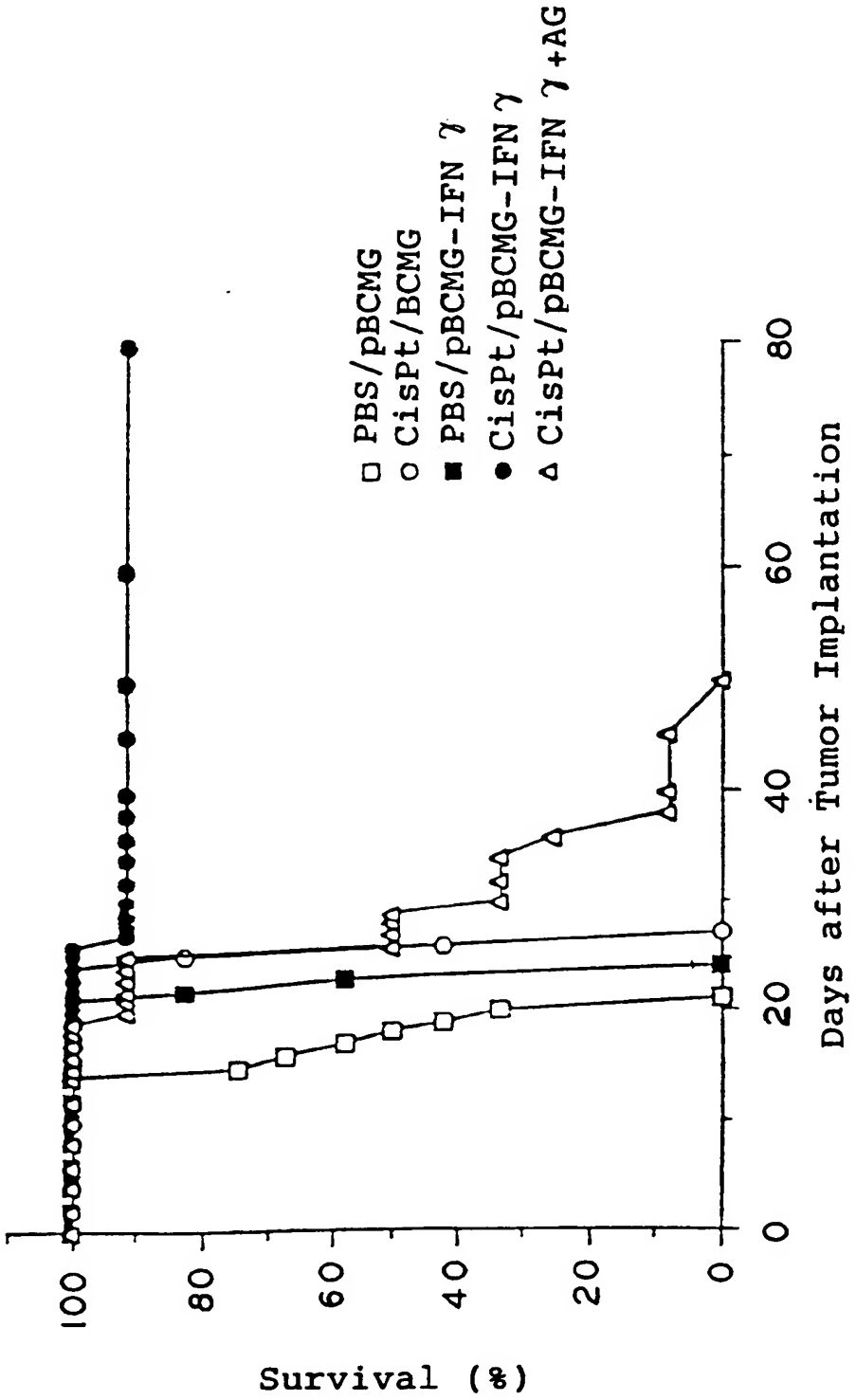

□ PBS/pBCMG
○ CisPt/BCMG
■ PBS/pBCMG-IFN γ
● CisPt/pBCMG-IFN γ
△ CisPt/pBCMG-IFN γ +AG
Survival (%)
100
80
60
40
20
0
0
20
40
60
80
Days after Tumor Implantation

FIG. 3B

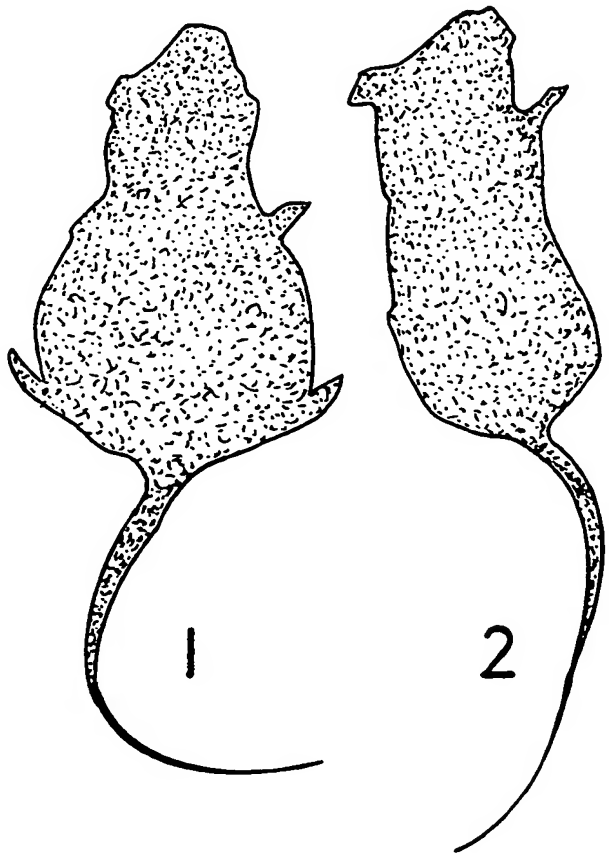
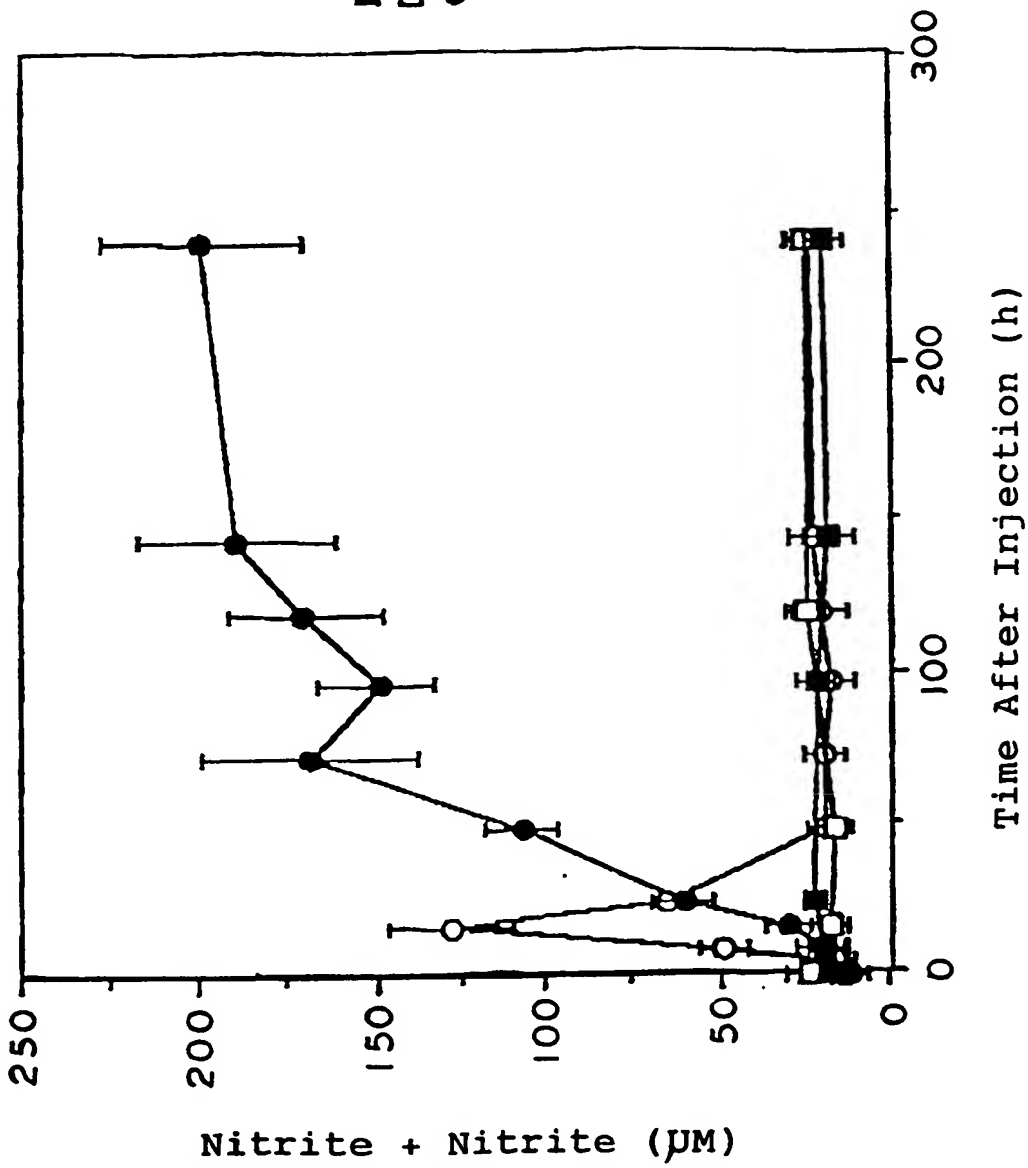


FIG. 4


CisPt/PBS
CisPt/Empty Vector
CisPt/IFNγ- Expression Vector
CisPt/IFNγ
Nitrite + Nitrite (μM)
Time After Injection (h)
0
50
100
150
200
250
0
100
200
300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06977

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 33/24; A01N 33/24, 43/04, 43/64, 63/00

US CL : 424/649, 93.1; 514/242, 44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/649, 93.1; 514/242, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, CAPLUS, APS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SYLJUASEN et al, Sensitization of renal carcinoma to radiation using alpha interferon (IFNA) gene transfection. Radiation Research. 1997, Vol. 148, pages 443-448, especially page 446.	1-20
Y	CONNOR et al, Regression of bladder tumors in mice treated with interleukin 2 gene-modified tumor cells. J. Exp. Med. April 1993, Vol. 177, pages 1127-1134, especially page 1132.	1-20
Y	BENZ et al, Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER/neu. Breast Cancer Research and Treatment. 1992, Vol. 24, pages 85-95, especially pages 93 and 94.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 JUNE 1998	10 AUG 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer LIN SUN-HOFFMAN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

FIG. 1A

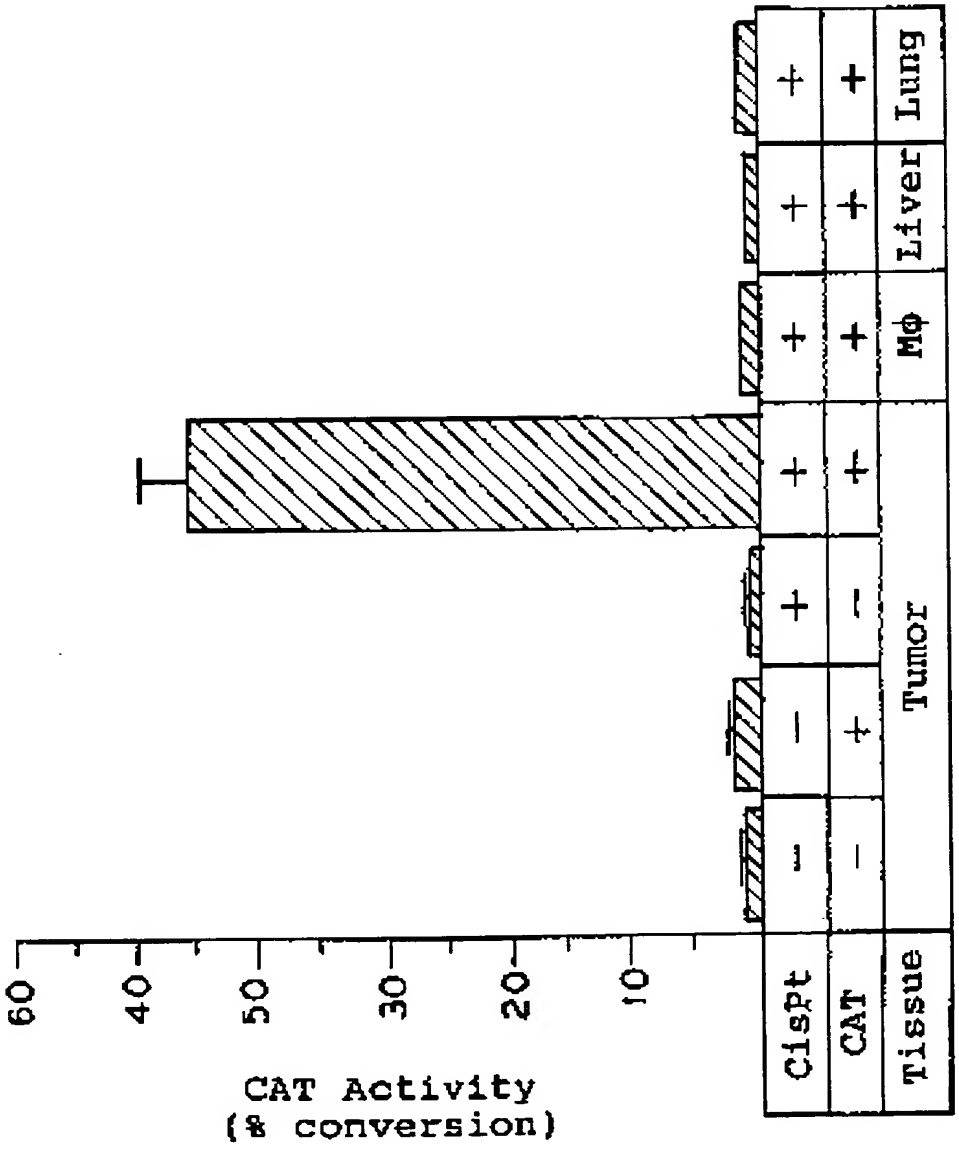

CAT Activity
(% conversion)
60
40
50
30
20
10
Tissue
CAT
CisPt
Tumor
Mφ
Liver
Lung
-
+

FIG. 1B

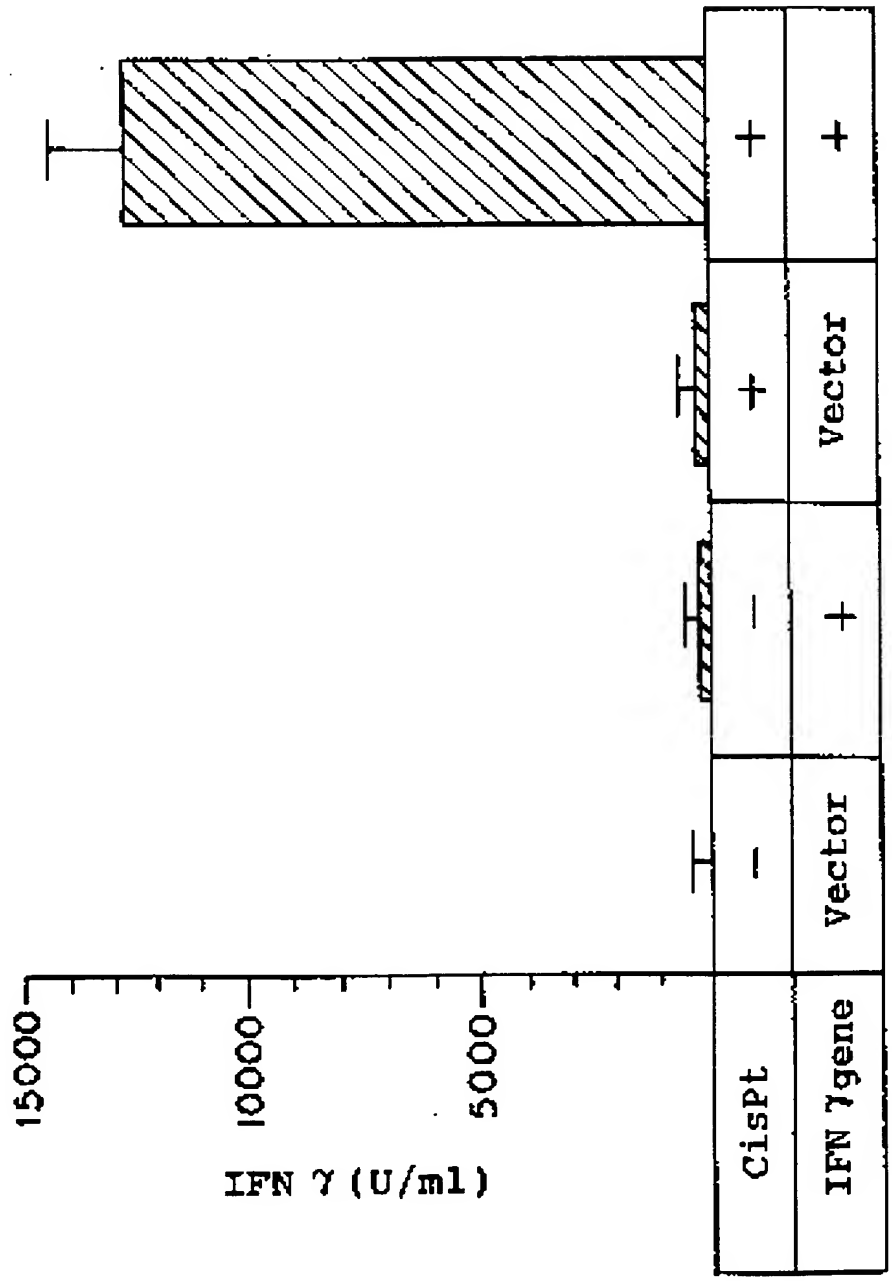
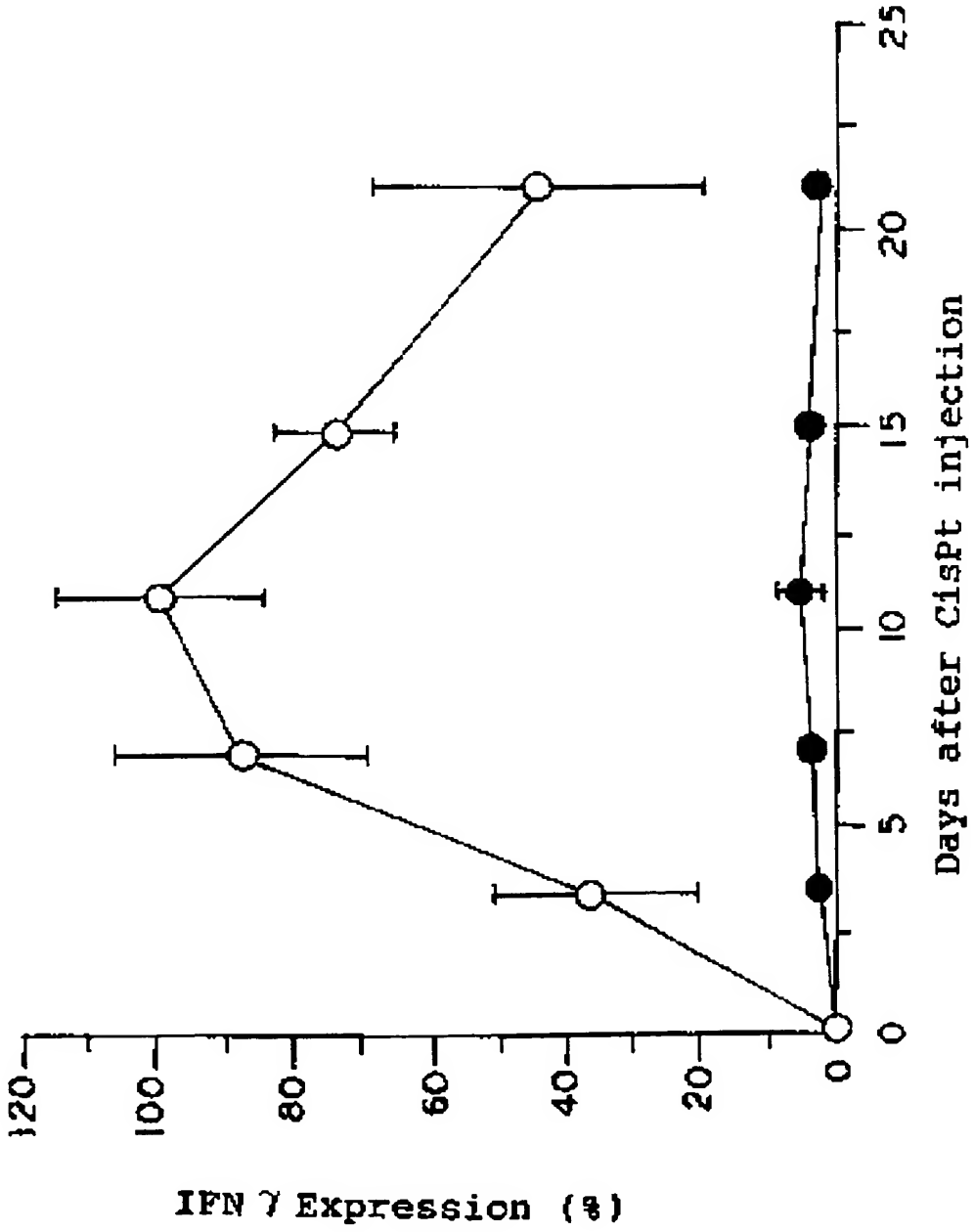


FIG. IC


IFN γ Expression (%)
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100
80
60
40
20
0
0
5
10
15
20
25
Days after CisPt injection

FIG. 1D

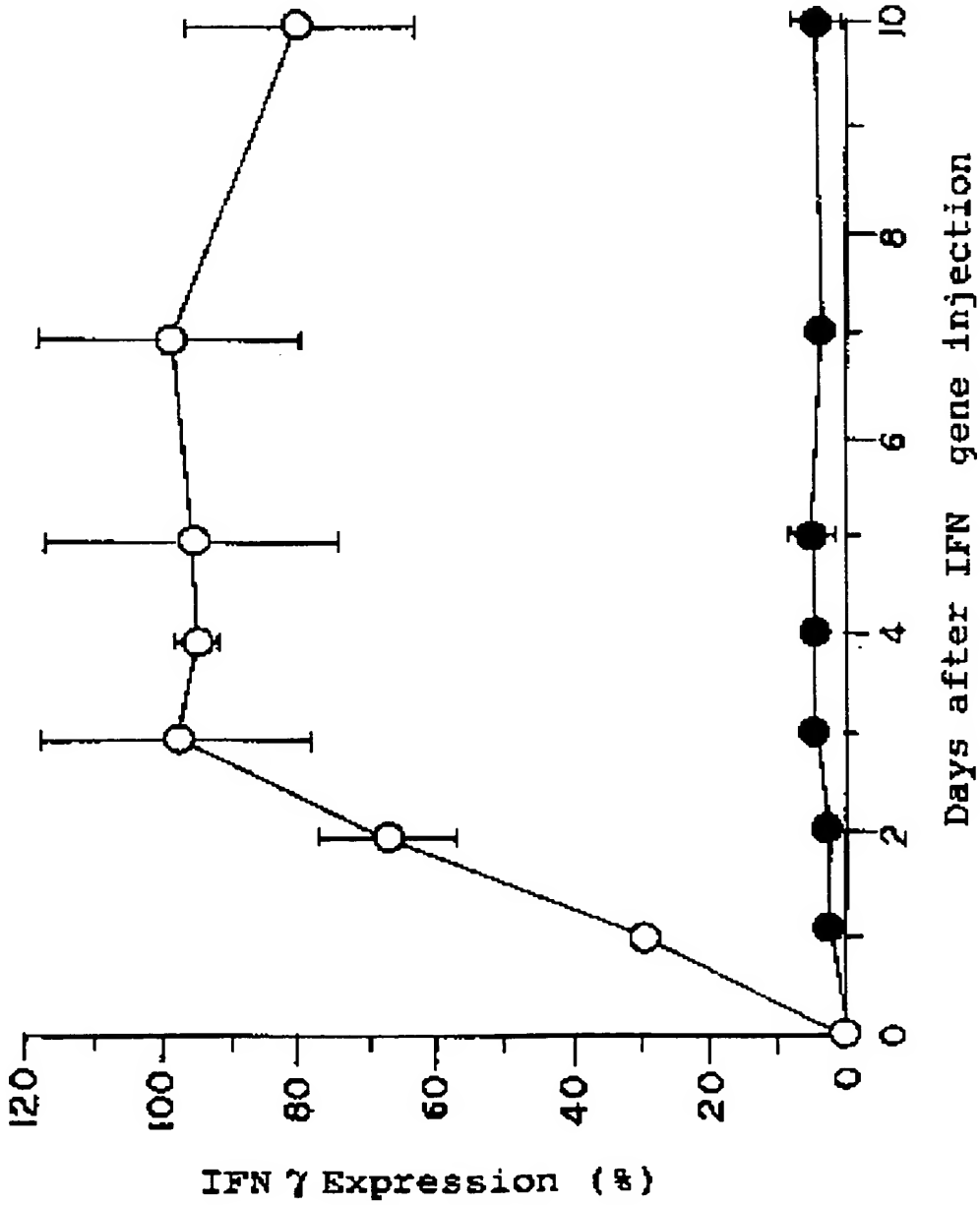

IFN γ Expression (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
Days after IFN gene injection

FIG. 2A

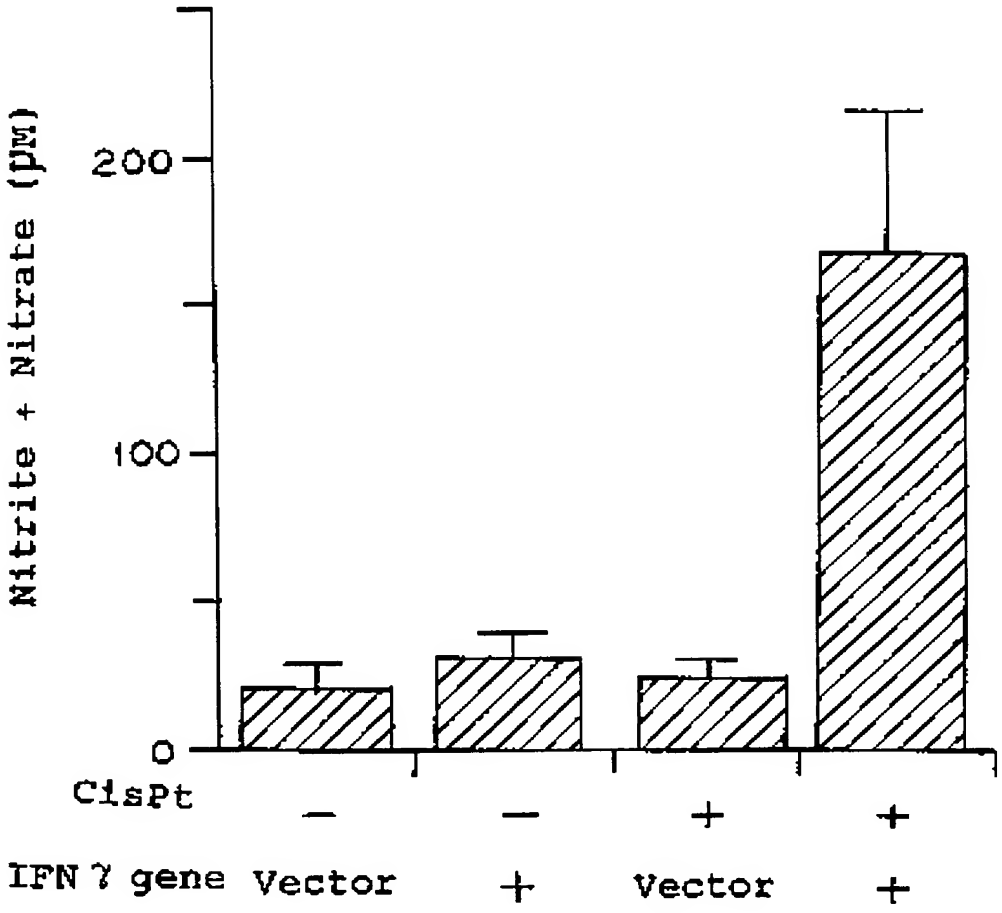


FIG. 2B

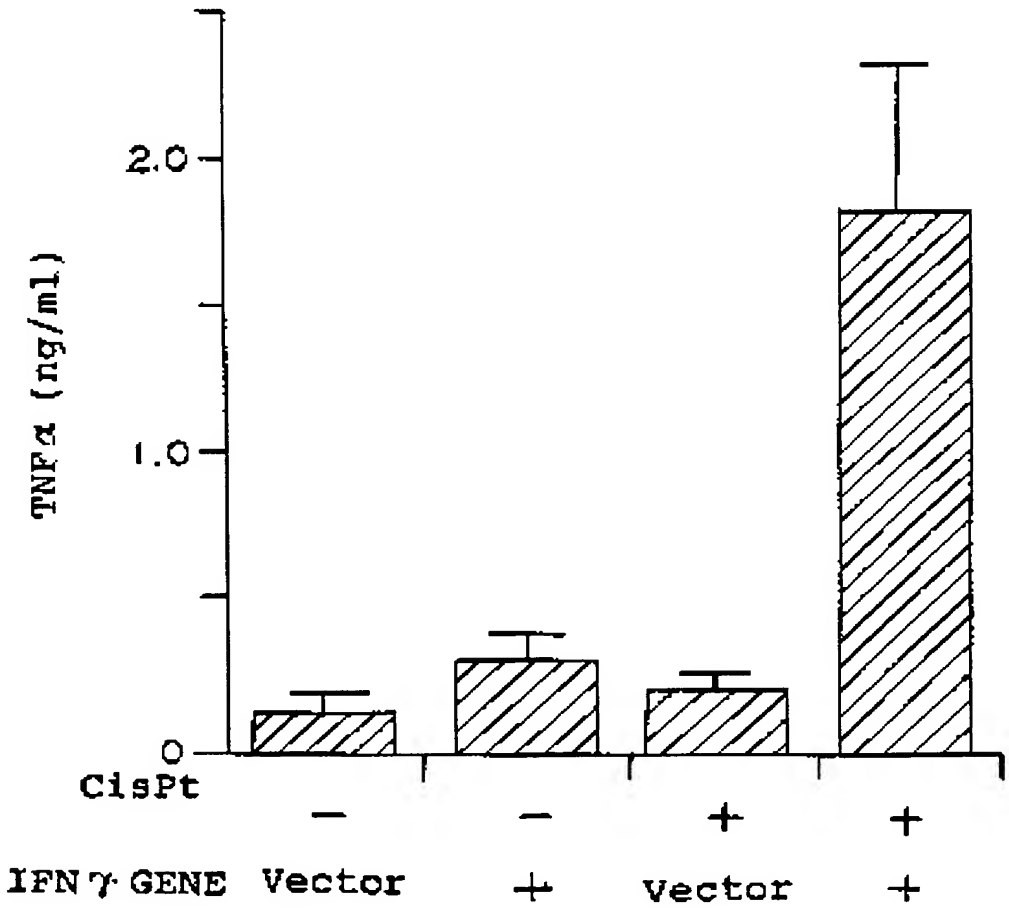


FIG. 3A

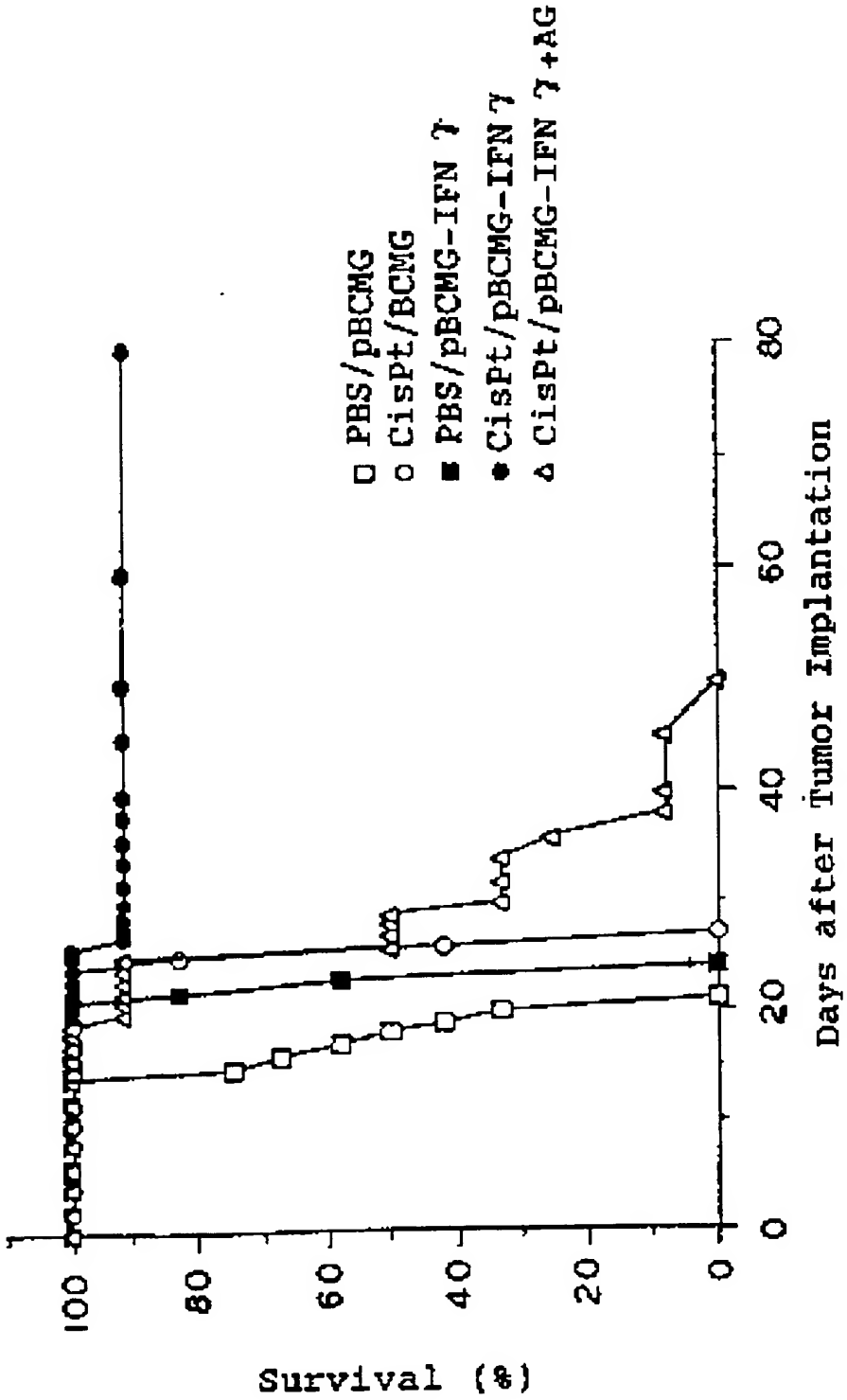

□ PBS/pBCMG
○ CisPt/BCMG
■ PBS/pBCMG-IFN γ
● CisPt/pBCMG-IFN γ
△ CisPt/pBCMG-IFN γ+AG
100
80
60
40
20
0
Survival (%)
0
20
40
60
80
Days after Tumor Implantation

FIG. 3B

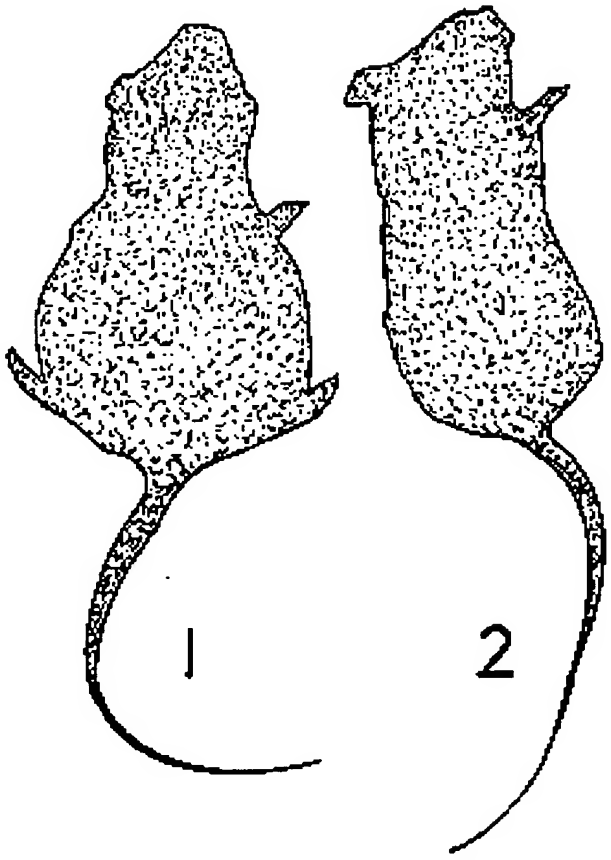
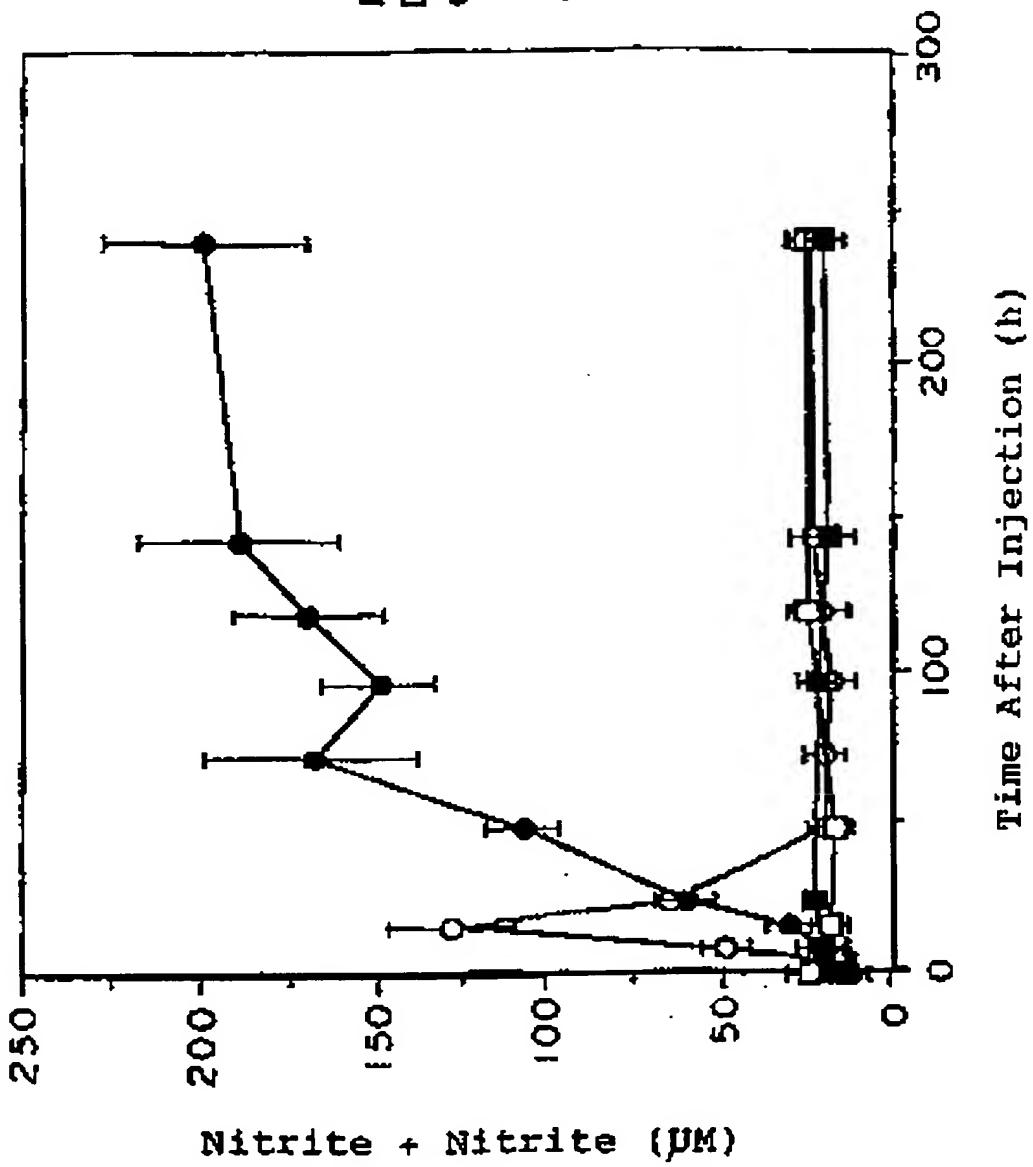


FIG. 4


CisPt/PBS
CisPt/Empty Vector
CisPt/IFNγ- Expression Vector
CisPt/IFNγ
Nitrite + Nitrite (μM)
Time After Injection (h)
0
50
100
150
200
250
0
100
200
300